Variability of Hormonal Stress Markers Collected from a Managed Dolphin Population

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LONG-TERM GOALS

Quantifying physiological indicators of stress in wild marine mammals and the interrelationships between different stress markers can be used to estimate the impact of anthropogenic stressors on marine mammal populations. The United States Navy, as part of its environmental stewardship, can utilize stress markers to assess the acute and chronic impacts that its actions might have on marine mammals. This approach would permit better mitigation of potential impacts and ensure that Navy activities do not come at a deleterious cost to marine mammal populations.

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OBJECTIVES

The objectives of this effort are to: 1) determine the variation in corticosteroid hormones, thyroid hormones, and catecholamines within a dolphin population relative to seasonality, time of day, gender, age and reproductive state; 2) assess relationships between serum corticosteroid levels and levels found in other matrices (i.e. biological samples), including feces, saliva, and blubber; 3) and to perform adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH) challenges to characterize the activation of the hypothalamic-pituitary-adrenal (HPA) and hypothalamic-pituitary-thyroid axes across multiple matrices, respectively.

APPROACH

Task 1 – Seasonal variations in hormones across multiple matrices
Regular sampling from different matrices (e.g. blubber, blood, feces) will be collected from the U.S. Navy Marine Mammal Program (MMP) dolphin population over the course of a year. Subject dolphins will be split into categories based upon age: 5-15 years, 16-25 years, and 26-35 years. All efforts will be made to structure the subject pool such that ten animals will be drawn from each category with a roughly equal number of male and female subjects. Each animal will be sampled bi-weekly throughout the year for blood and feces. The same matrices will be collected from pregnant females depending on the availability of subjects (there are typically 2-5 pregnant females available each year). A subset of animals will be selected for blubber biopsies, which will be conducted every month (pursuant to preliminary testing and approval of the veterinary staff).

Blood samples will be collected from dolphins through their voluntary participation. Blood collections will be made from the ventral fluke from the arteriovenous plexus. Attempts will be made to collect blood samples at the same time of day, approximately within a 3 hour window. Fecal samples will be collected by use of a suction catheter inserted into the anus of the dolphin. Fecal samples will be collected through voluntary cooperation with the dolphin and will be performed the day after the blood collection. Blubber biopsies will be collected with a 16g or 18g tissue biopsy needle and the condition and healing of the animal biopsied will be monitored daily following the procedure. The biopsy procedure will be attempted monthly to bimonthly, depending on animal health.

Serum samples will be processed for adrenocorticosteroids (ACTH, cortisol, aldosterone), catecholamines (epinephrine, norepinephrine), and thyroid hormones (T3 and T4) via radioimmunoassay (RIA). Radioimmunoassay methods have previously been validated for cortisol and aldosterone in this species (Houser et al., in press). Parallel processing of serum catecholamines will be performed via high-performance liquid chromatography (HPLC) to assess variability in the measurement methods available for these hormones. The use of HPLC is the standard approach to catecholamine measurement from blood samples and will be used to validate the RIA methods and determine the quantitative differences and limitations of the two assays.

Metabolites of cortisol, aldosterone and thyroid hormone will be extracted from fecal samples and measured via RIA using methods described in Wasser et al. (2000, in review). Cortisol and thyroid fecal metabolites have already been validated for killer whale feces. The Wasser lab has also tested excretion of many other steroids and found most to be predominantly excreted in scat (Wasser et al 1994, 1996, 2000). Thyroid measures will focus on T3 because I131 ingestion studies showed that thyroid hormone was excreted in feces almost entirely as T3 with very little T4 in two domestic dogs, and similarly only immunoreactive T3 was primarily found in killer whale feces with lesser amounts of

T4 (Wasser et al., in review). T3 was also found to be the primary bioactive thyroid hormone excreted in feces following TSH challenges in Steller sea lion (Keech et al. 2009).

A multi-step biphasic organic solvent extraction with be used to isolate the corticosteroids from the blubber tissue (Kellar et al., 2009). The hormones will be measured using a commercially available enzyme immunoassay (EIA) and parallel processing via HPLC will be used to verify method performance.

Task 2 – Diurnal variation in hormone production

Diel variability in corticosteroids, thyroid hormones, and catecholamines should ideally be determined as a number of these hormones show cyclic variations that could potentially lead to misleading conclusions as to the stress level of animals singly sampled in the wild. Given the practical constraints of working with MMP dolphins at night, these hormones will be assessed for diurnal variation during the second year of the study with the goal of assessing changes that occur between dawn and dusk.

Ten dolphins will be selected for repeat testing throughout the year; every effort will be made to ensure that five adult males and five adult females are selected. Blood samples will be collected from the dolphins at biweekly intervals via voluntary venipuncture of the arteriovenous plexus on the ventral fluke. Paired blood samples will be collected on the day of sampling. The initial samples will be collected first thing in the morning (~0700) and at noon. At the next sample period, samples will be collected first thing in the morning and late in the afternoon (~1700). The timing of paired samples will alternate according to the biweekly schedule. Blood samples will be processed via RIA and HPLC as described under Task 1. Similar analyses will be conducted on serially collected scat of these 10 individuals over the same 24 hr period and a second 24 hr period one week later when not being sampled for blood.

Task 3 – Adrenocortical sensitivity

Adrenocortical sensitivity and the relationship between activation of the HPA axis and reflection of this activation in serum and other matrices will be determined during the second and third years of the study. The information from this assessment will allow researchers to better understand the temporal and quantitative relationship between hormones measured in matrices likely to be collected from wild animals, namely feces and blubber, and that circulating in the blood stream.

Three animals will be selected in the first year of the study for determining the appropriate dose of ACTH required to sufficiently elevate the corticosteroids in serum and other matrices. ACTH slow-release gel will be intramuscularly implanted to permit time-controlled and sustained release of ACTH. Implantation of the gel will be performed by MMP or NMMF veterinarians and the animal will be monitored for the first hour following the injection. Repeat blood samples will be taken daily for several days to determine the relationship between the time course of serum corticostroid increase and the ACTH dose administered. Serum samples will be processed for corticostroids as described in Task 1. Based upon results of the pilot study, a schedule will be determined for the collection of samples from other matrices (feces and blubber) that will be tested during the second and third years of the study. Five dolphins will be selected during the second and third years of the study for ACTH challenges. For each of the dolphins, blood, feces and blubber samples will be collected according to the sampling schedule determined during the first year of the study. Feces and blubber will be processed for hormone concentrations as described in Task 1.

The dynamics of cortisol in blubber potentially mean that increased plasma cortisol after administration of ACTH treatment may not elevate plasma cortisol for a sufficient period of time to increase blubber cortisol levels to a noticeably elevated state. If it is found that the ACTH challenge does not sufficiently increase and sustain serum cortisol to a point where the increase is reflected in the blubber, a pilot study will conducted in which a dolphin is fed fish containing cortisol pellets. The FDA approved oral tablets will contain hydrocortisone (cortisol) in concentrations not to exceed 25 mg per tablet. For the first pilot study, 25 mg of cortisol will be fed to the dolphin in fish at six hour intervals over a 10 day period to attempt to raise and maintain the serum cortisol levels. Voluntary blood samples will be collected at 0, 1, 2, 4, 6 and 10 days to determine if serum cortisol levels are elevated and sustained. Based on the results, the pilot will either be repeated with an increase in the cortisol dosage or a blubber biopsy schedule will be determined. Cortisol will be increased by 25 mg per feeding for the second pilot, and again by 25 mg per feeding for the third pilot, provided the subsequent pilot procedures are needed. No more than three biopsies will be taken over the course of the 10 day period and no more than three animals will be used in the pilot. Provided the procedure adequately raises cortisol levels, the process will be repeated in the following year with five bottlenose dolphins. Since abrupt cessation of cortisol treatment has been observed to cause side-effects, dolphin subjects will be weaned off of the cortisol over a period of four days (25% reduction per day).

Task 4 – Thyroid challenges

Thyroid hormones (thyroxin, T4 and triiodothyronine, T3) are released from the thyroid gland and are responsible for regulating the metabolism of an animal and affecting the activity of other stress hormones via permissiveness. Thyroxin is the more abundant of the two thyroid hormones in circulation and the metabolic parent hormone. However, the bioactive form is largely T3, which is roughly eight times more potent than T4 (Tomasi 1991). Thyroid hormone production is known to be affected by stress, which can lead to conditions of both hypo- and hyperthyroidism. Persistent elevated or diminished levels of these hormones are known to lead to pathophysiological conditions that can ultimately impact important life history functions.

Thyroid hormones are produced in response to the presence of thyroid stimulating hormone (TSH), which is a peptide hormone produced in the anterior pituitary gland. Thyroid stimulating hormone is itself produced in response to the action of thyrotopin-releasing hormone (TRH), which is produced in the hypothalamus. Assessing the responsiveness of the production pathway to acute elevations of TRH, i.e. a hormone challenge, is one means by which pathway responsiveness and activity of TRH can be quantified at different levels of the synthesis pathway.

Three dolphins will be given an exploratory TRH challenge to determine the optimal dosing and sampling schedule. A pre-test blood draw will be collected from the dolphin while it is in its enclosure. The dolphin will then be removed from the water to a location on the pier on in the veterinary clinic that is deemed suitable for the procedure by the attending veterinarian. A bolus injection of 50 µg of TRH will be intravenously administered via the venous plexus of the fluke. Blood samples will then be collected every 15 minutes for a period of 4 hours. Dosages of TRH will be adjusted for the second and third animal in the pilot study following analysis of the blood samples collected with the first challenge. Dosages will not exceed 200 µg, which is the common dosage for human TRH tests. Provided that the pilot studies show responses to the challenges without negative impact to the subjects, eight individuals will be submitted to the TRH challenge. Baseline blood and fecal samples will be collected prior to the first injection and blood collections will be performed as described for the pilot studies. All fecal samples will be collected for 96 hrs following injection. Blood and fecal samples will be assayed as described in Experiment 1.

WORK COMPLETED

Task 1 – Seasonal variation in stress hormones
A group of 30 bottlenose dolphins were identified from within the U.S. Navy Marine Mammal Program's animal population that could provide biweekly blood samples over a period of a year. However, due to limitations on the number of dolphins available in each age category, the following distribution of animals was obtained:

Age (yrs)	Male	Female
5-15	6	4
16-25	3	3
25+	7	7

Each of the dolphins was scheduled for a biweekly blood sample with an attempt at a fecal collection the following day. Four animals were identified for monthly blubber biopsies to be collected on the same day following the blood collections.

Voluntary blood collections were attempted on each animal on a biweekly basis. As of 16 September, 147 blood collection attempts were scheduled. Of these, 141 collections were made. Blood collection attempts failed on four animals and scheduled collections were not made on two other animals due to conflicting factors. All collections were made between 0700 and 1000 in the morning. Sufficient blood was obtained to test for all of the corticosteroids, thyroid hormones, and the catecholamines. All samples were centrifuged immediately after collection and the plasman or serum frozen immediately on dry ice or in liquid nitrogen. All samples were stored at -80° C until they could be processed.

Blubber biopsies were collected with a 18g Biopinch biopsy needle. The biopsies were collected approximately 12-14 cm below the posterior insertion of the dorsal fins. Two or three biopsies were taken each sample period to ensure that sufficient blubber was obtained for analysis. During the first month of the study, biopsies were collected on two animals. The sample size was expanded to four animals in the second month. All samples were stored at -80° C until they could be processed. Fecal collections were attempted the day after each blood collection. Of the 144 attempts at collection, 111 fecal collections were succesful. Fecal collections were also stored at -80° C until they could be processed.

RESULTS

No samples have been processed as of the writing of this report. The sampling protocol, though challenging, has proven successful and the animals have been robust in their willingness to participate. None of the sampling protocols have produced any deleterious effects in the dolphin subjects.

IMPACT/APPLICATIONS

The ability to identify stress markers relative to monitoring the health of marine mammal populations is critical to understanding the impact of anthropogenic activities upon those populations. The baseline characterization of stress marker variation in dolphins as a function of seasonality, gender, age, and reproductive status is important to assessing measurements made in wild dolphins. Information on levels and dynamics of stress markers between different matrices will provide better estimates of the overall condition of marine mammals sampled in the wild from either blubber biopsies or fecal collections. In addition, an understanding of the function of the HPA and HPT axis will provide fundamental information on the stress response in these marine mammals, which may differ significantly from that of the terrestrial mammals from which most of our understanding is based.

RELATED PROJECTS

Project: Pathophysiology of Stress in Wild and Managed-Care Bottlenose Dolphins
PI: Pat Fair

This project looks at numerous markers of stress in a wild population of marine mammals and compares them to animals under managed care in order to quantify and qualify the impact of environmental stressors on wild dolphins. The dolphins under managed care are from the Georgia Aquarium and the Navy Marine Mammal Program. A subset of the dolphins used in the current study (PI – Houser) are used as the semi-domesticated comparison (i.e. they are under managed care but are exposed to pathogens and contaminants of the ocean).

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